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EXPRESSION AND RESPONSE TO HYPOXIA OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN RAT AND RABBIT TISSUES

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INTRODUCTION

VEGF is a potent and specific endothelial cell mitogen and an important regulator and inducer of angiogenesis. Recent finding of capillary budding in hypoxic rat brain suggested that hypoxia might initiate angiogenesis in brain. As a key factor of angiogenesis, VEGF is thought to be potent in the basement membrane destruction and leakage. It can enhance microvascular permeability with a potency some 50,000 times that of histamine,[11] which is likely to elicit hypoxic cerebral edema. The pathologic findings of cerebral thrombosis and petechial hemorrhages[12] in high altitude cerebral edema are also consistent with VEGF physiological effects such as induction of thromboplastin activity[5] and stimulation of von Willebrand factor release from endothelial cells.[2] We surmise that VEGF may contribute to high altitude cerebral edema (HACE). Here we report the expression and response to hypoxia of VEGF mRNA, VEGF protein and its receptor (flt-1) in hypoxic tissues.

METHODS

Animal Experiments

16 rabbits (Lepus New Zealand white, 3~4 kg, either sex) and 32 adult Sprague-Dawley rats (Hilltop Strain, 280–300 grams, either sex) were used in the experiment. Rabbits and rats were housed in an aquarium with a plastic cover. A continuous fresh gas flow was supplied to the chamber. During the period of hypoxia, the oxygen concentration was progressively lowered from 9.0% to 6.0% while the carbon dioxide concentration was kept below 1%. Rabbits and rats were supplied with food and water and kept in normal circadian rhythms. In rat experiment, 8 rats were housed in the same chamber for each group to insure equal hypoxic exposures for all animals. The rats were harvested sequentially at 8 experimental time points

which were 0, 3, 6, 12 hours and 1, 2, 3, 6 days after hypoxia. The procedure was repeated for a total of 4 runs of 8 rats each. General appearance, activity, response to stimuli and intake and excretion were recorded daily. Oxygen and CO_2 concentration were continuously monitored using the laboratory PDP 11/44 computer and Perkin Elmer mass spectrometer. The rats were anesthetized with halothane followed by decapitation. Brains, kidneys and lungs were quickly removed, frozen with liquid nitrogen and stored at -80 °C.

Northern Blot Hybridization

Total RNA was isolated from 100 mg pieces of rabbit or rat tissues using a single-step method. Tissue was homogenized in 1 ml of RNA STAT-60 (TEL-TEST"B", Inc. Friendswood, TX) using polytron homogenizer. The total RNA was extracted with chloroform and precipitated with isopropanol followed by washing with 70% ethanol. The RNA pellet was dissolved in TE buffer (pH 7.5). 10 µg of total RNA was denatured and subsequently electrophoresed on a 1% agarose gel containing 2.2M formaldehyde in 1X MEA. RNA was transferred to Hybond N membranes (Amersham, Bucks, UK) in 20X SSPE. Blots were cross-linked and prehybridized at 50 °C for 1 hour in prehybridization solution (1M NaCl, 1% SDS), then hybridized at 65 °C overnight in hybridization solution (1M NaCl, 1% dextran sulfate and 100 µg/ml denatured salmon sperm DNA). The rat VEGF cDNA and flt-1 cDNA were labeled with ^{32}P dCTP using Redprime DNA Labeling System (Amersham). Blots were exposed to X-ray film at -80 °C overnight. All the blots were stripped and reprobed for β-actin RNA probe (Ambion Inc., Austin) in order to permit loading and blotting differences between lanes to be compensated. The intensity of the signals was quantified by a scanning densitometer.

Western Blot Analysis

100 mg of rabbit or rat brain tissue were homogenized thoroughly in 1 ml lysis buffer (0.01 M Tris/HCl, pH 7.6, 0.1 M NaCl, 0.1 mM DTT, 0.001 M EDTA, 0.1% NaN_3, 1 µg /µl Leupeptin, 100 µg /ml PMSF, 1 µg /µl Aprotinin, 1% NP-40). The extracts were centrifuged to remove particles. 40 µg of total protein dissolved in sampler buffer containing 2-mercaptoethanol was loaded into each individual lane. 20 µl of 1µg/ml rh-VEGF was used as a positive control. The proteins were separated by 10% SDS-PAGE at 4 °C and then transferred onto Hybond ECL nitrocellulose membrane (Amersham). The blot was blocked with 10% solution of dry milk overnight, incubated with polyclonal antibody against recombinant human VEGF (Santa Cruz Biotechnology, CA) at a dilution 1:500 in 10% dry milk in TBS/0.1% Tween for 1 hour at room temperature with gentle agitation. Subsequently, the filter was rinsed several times and incubated with anti-rabbit IgG horseradish peroxidase conjugate (Vector Laboratories, Burlingame, CA) at a dilution of 1:20,000 in 1% milk for 1 h at room temperature. The detection of immunoreactive proteins was done using the ECL Western Blotting Detection System (Amersham). The membrane was exposed to Hyper film ECL (Amersham) at room temperature for 5 min. to 2 hours.

RESULTS

Tolerance to Hypoxia

Ambient O_2 concentration was reduced to about 9% initially, and over the next hours and days, progressively, in response to activity, to as low as 6%. O_2 concentration was re-

duced until the animals showed little activity and a decreased intake of food and water. During the first 3 days the tolerated level of O_2 appeared to stabilize or in some cases, rose. Rabbits could survive at least 14 days with as low as 6% oxygen. However, rabbits seemed to be less tolerant of hypoxia than rats. During exposure, 6 out of 16 rabbits died while only 2 out of 34 rats died. All deaths occurred during the first three days of hypoxia. Brain surface vascularity appeared more congested in the hypoxic animals than in controls. Histopathologic examination of brain from one rabbit after 6 days of hypoxia revealed petechial hemorrhages throughout the brain.

Expression of VEGF mRNA in Hypoxic Brains of Rats and Rabbits

After exposure to hypoxia for as little as 3 hours , VEGF mRNA levels were remarkably increased in rat brain tissue, reaching a maximum at approximately 12 hours. Brain VEGF mRNA increased about 3 fold within the first 24 hours. Enhanced VEGF mRNA persisted for at least 6 days but then fell despite constant or increasingly severe hypoxia. RNA from rat brain exhibited two hybridization signal bands for VEGF at approximately 3.9 kb and 4.7 kb. Both bands were upregulated by hypoxia. In normal rat brain, VEGF mRNA expression in cerebellum was higher than in cerebral cortex. The response of VEGF mRNA to hypoxia was stronger in cerebral cortex than in cerebellum (Figure 1. A). Hybridization patterns with RNA for β-actin were used as an index of the amount of total RNA applied to each lane. The upregulation of VEGF expression after hypoxia can also be seen in rabbit. The maximal effect of hypoxia occurred in 16 hours. The enhancement was then decreased but still remained higher after 6 days of hypoxia (Figure 1. B).

Induction of VEGF Protein Production in Rat Brain Tissue by Hypoxia

SDS-PAGE was performed under reducing conditions. A protein band at 23 KDa was detected in both cerebral cortex and cerebellum using polyclonal antibody against human VEGF165. VEGF in rat brain was not increased until 12 hours of hypoxia, although the VEGF mRNA increased as early as 3 hours in hypoxia (Figures 2 and 3). No obvious difference in the production of VEGF protein was observed between cerebellum and cortex even though the VEGF mRNA was mainly located in cerebellum in normal rat brain.
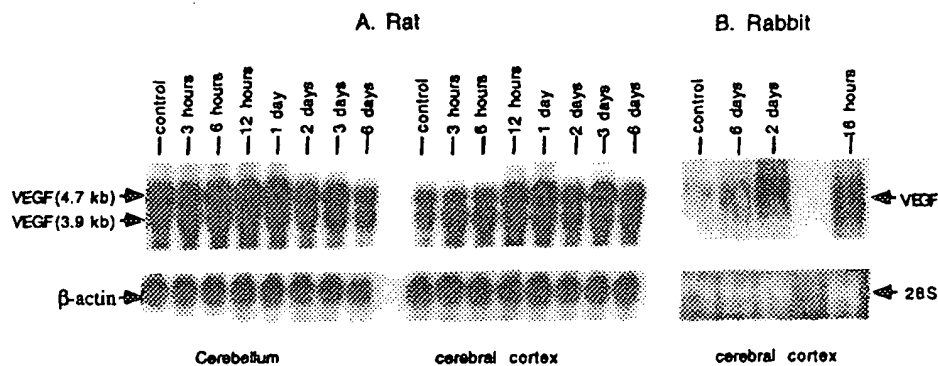

Figure 1. Expression and response to hypoxia of VEGF mRNA in rat brain (A. cerebellum and cerebral cortex) and rabbit brain (B. cerebral cortex). Rats and rabbits were exposed to 9% to 6% oxygen for different periods from 3 hours ~ 6 days. 10 μg of total RNA was loaded in each lane. β-actin (A) hybridized with blots or 28S (B) in ethidium bromide stained gel were used as a reference for normalizing the total RNA loading.
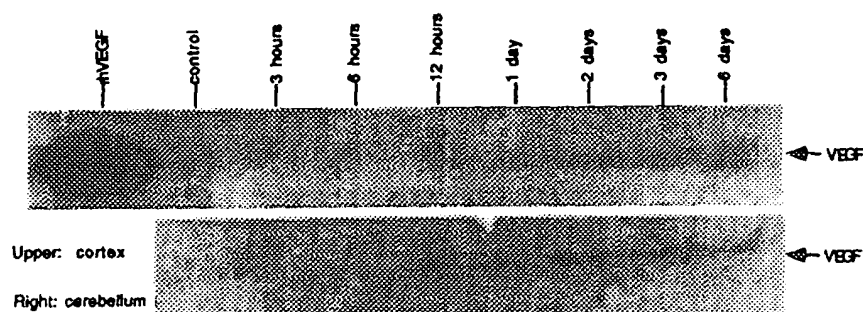
Figure 2. Western blot analysis of brain tissue extract (cerebral cortex and cerebellum) of normal rats and rats exposed to hypoxia for 3 hours ~ 6 days. 40 µg of total protein resolved under a reducing condition was loaded in each lane and separated by 10% SDS-PAGE. Nitrocellulose blot was immunostained with polyclonal antibody against recombinant human VEGF.

Upregulation of VEGF Receptor (Flt-1) by Hypoxia in Rabbit and Rat Brains

Along with VEGF mRNA and VEGF protein, Flt-1, one of the two VEGF receptors, was also induced by hypoxia. The expression of flt-1 mRNA in normal rat was very low and upregulated after 3 days of hypoxia. The level of flt-1 mRNA in rabbit brain was relatively higher than in rat brain, it also increased after 3 days of hypoxia (Figure 4).
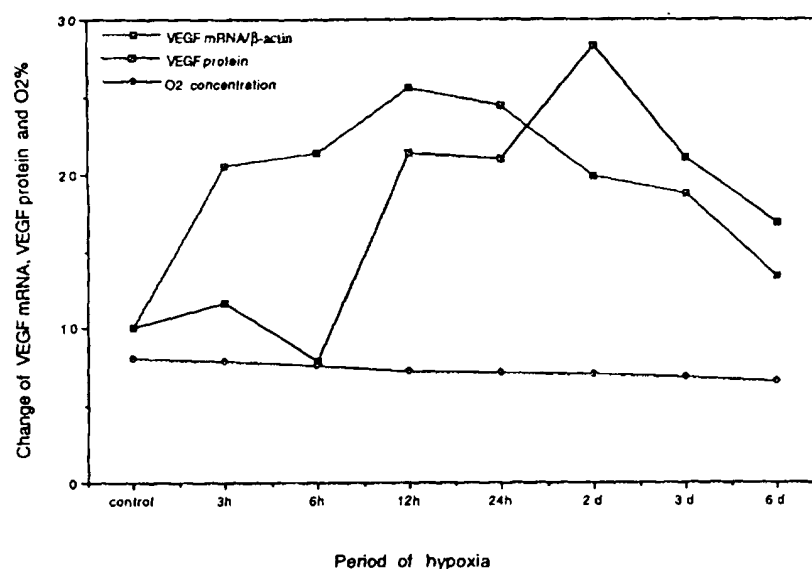
Figure 3. Effects of hypoxia on VEGF mRNA expression and VEGF protein production in rat brain. VEGF mRNA optical density was corrected for β-actin. The scales of VEGF mRNA/ β-actin and VEGF protein of control rats were normalized to 10. The changes of VEGF mRNA/ β-actin and VEGF protein after different periods of hypoxia shown on the horizontal axis were relative values compared with normal control.
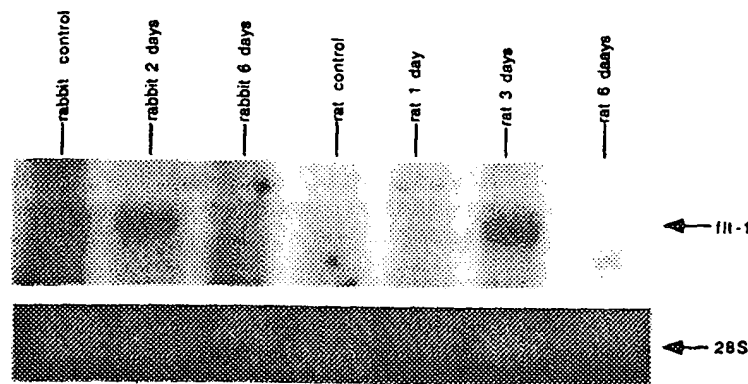
Figure 4. Expression of Flt-1 mRNA in rabbit and rat brain tissue after hypoxia. 10 μg of total RNA extracted from normal or hypoxic brains of rabbits or rats was loaded onto each lane. The blot was hybridized with flt-1 cDNA probe. 28S ribosome in ethidium bromide stained agarose gel was used as an index of the amount of total RNA applied to each lane.

Expression of VEGF mRNA in Rat Lung and Kidney after Hypoxia

The upregulation of VEGF mRNA in rat kidney was similar to that in rat brain tissue. Both 3.9 kb band and 4.7 kb band of VEGF mRNA were enhanced after 3 hours of hypoxia, peaking at 12 hours of hypoxia (Figure 5). VEGF mRNA expression in normal lung was abundant. No obvious upregulation was observed in lung after hypoxia.

DISCUSSION

The expression of VEGF mRNA was increased within 3 hours of hypoxia in rat brain and kidney, suggesting that VEGF is an early regulatory factor in response to hypoxia. Simi-
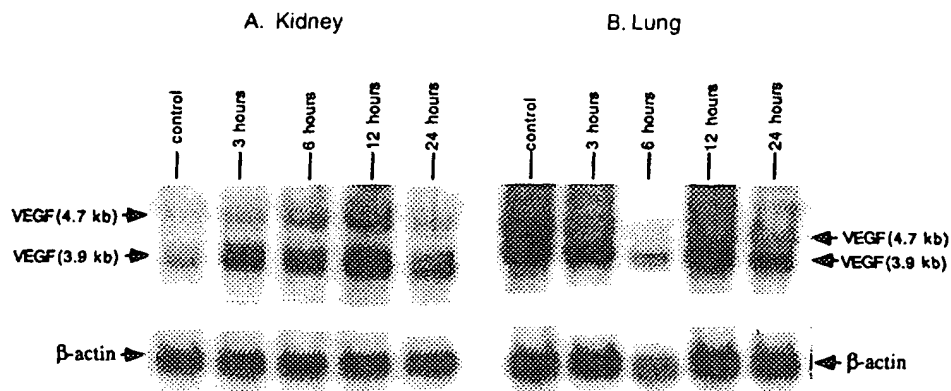
Figure 5. Northern blot analysis for VEGF mRNA expression in rat kidney and lung. The rats were exposed to hypoxia for 3, 6, 12 or 24 hours. 10 μg of total RNA extracted from kidney or lung was loaded each lane. Blots were sequentially hybridized with VEGF cDNA and β-actin probe which was used to correct for variation in total RNA loading between lanes.

lar results were observed in rat heart muscle.[6] The production of VEGF protein in rat brain was induced after 12 hours of hypoxia as a result of increased VEGF gene expression during hypoxia. VEGF mRNA expression peaked at 12~24 hours after hypoxia while the production of VEGF protein reached its maximum at 48 hours of hypoxia, then both VEGF mRNA and protein declined despite continuous hypoxia. This time course indicates that VEGF is also a transient regulatory factor in response to hypoxia. LaManna et al have demonstrated capillary budding in rat brain within one week of hypoxia, but no more budding can be seen after 3 weeks of hypoxia even though the increased capillary length and density were observed.[9] Since VEGF is thought to play an important role in triggering angiogenesis. The time course of VEGF induction is consistent with above observations, suggesting that VEGF may be involved in the capillary budding in the early phase of angiogenesis in hypoxic brain tissue. With its permeability enhancing properties, VEGF may act as a mediator in the process of hypoxic cerebral edema, assuming the role of a link between tissue hypoxia and an angiogenesis response. However, direct evidence for a causative relationship between expression of VEGF and brain edema must await additional studies. Better methods are needed to evaluate brain edema and/or petechial hemorrhage in order to quantify possible effects of use of VEGF antibody or other VEGF blocking methods.

VEGF may exist in four different homodimeric molecular species due to alternative splicing of mRNA, each monomer having 121, 165, 189, or 206 amino acids.[7] The VEGF isoforms have different properties in vitro, which may determine their function in vivo. All four molecular species of VEGF can promote microvascular permeability.[7] Our results revealed two VEGF mRNA transcriptions, 3.9 kb and 4.7 kb, in rat brain tissue, kidney and lung. The bands at 3.9 kb and 4.7 kb seem to represent the $VEGF_{165}$ and $VEGF_{189}$.[10] Western blot analysis under reducing conditions showed a VEGF band at 23 KDa which is consistent in size with the monomer of $VEGF_{165}$. Both 3.9 kb and 4.7 kb of VEGF mRNA transcriptions were upregulated under hypoxia in rat brain and kidney.

In healthy adult rats, the relative abundance of VEGF mRNA is lung >> kidney $\geq$liver > brain >> spleen.[10] We found that VEGF was expressed abundantly in normal lung tissue but was not obviously upregulated within 24 hours of hypoxia. In contrast, the expression of VEGF mRNA in normal kidney was relatively lower, but it was increased under hypoxic exposure. VEGF upregulation in kidney may have a role in regulating renal glomerular permeability or may act as an mediator of renal glomerular dysfunction.[4] Krasney suggested that salt and water retention may contribute to the development of HACE.[8] The absence of VEGF upregulation in lung in our hypoxic rats is reasonable since the lung is the least hypoxic tissue in these experiments, whereas anoxic lung does express VEGF.[13] In rat brain, VEGF mRNA was expressed mainly in cerebellum[10] and in choroid plexus.[1] Our results showed that even the expression of VEGF mRNA is relatively higher in normal rat cerebellum than in cerebral cortex, the signal intensity of VEGF protein and the time course of its response to hypoxia showed no obvious difference between cerebellum and cerebral cortex.

There are two tyrosine kinase receptors: the fms-like tyrosine kinase receptor (flt-1) and the tyrosine kinase receptor (KDR). Both flt-1 and KDR are expressed exclusively in endothelial cells. Studies have disclosed that the expression of the flt-1 and KDR transcripts can be upregulated by hypoxia both in vitro[3] and in vivo.[13] In our animal model, the flt-1 is upregulated at 2~3 days after hypoxia, coinciding with the maximum induction of VEGF protein in rat brain. Interestingly, the time course of the upregulation of VEGF and its receptor flt-1 was similar to that encountered in the development of symptoms and signs of HACE in humans during the first few days of continued high altitude hypoxic exposure.

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